Calibration Model for EPA QPCR Methods 1611.1 & 1609.1 (Enterococci) and Draft Methods C (E. coli) & B (Bacteroidales)

9th Biennial State of Lake Michigan/15th Annual Great Lakes Beach Association Joint Conference
Background

RWQC

• EPA recreational water quality criteria are intended to protect designated water body uses including swimming and other water contact activities.
• Previous 1986 RWQC were based on culturable fecal indicator densities:
  – *E. coli* (freshwater), enterococci (freshwater & marine)

2012 RWQC

• Based on previous and newer (2003-2007 NEEAR) epidemiology studies
• Updated criteria values for culturable *E. coli* (freshwater) & enterococci (fresh & marine) associated with 32 and 36 GI illnesses per 1000 beach water recreators
• Geo Mean, single sample statistical threshold values (STV - 95th percentile) and single sample beach action values (BAV - 75th percentile) are provided
• Corresponding values also provided for enterococci CCE by more rapid qPCR methods (EPA Methods 1611 and 1609)
• Provisions also made for site-specific evaluation of alternative methods (e.g. *E. coli* or Bacteroidales qPCR), procedures are described at:
Outline of presentation

- The basis of real time PCR quantification
- Absolute $C_T$ calibration models (standard curves)
- Comparative $C_T$ calibration models
- The “EPA” calibration model
  - Reporting units: TSC (target sequence copies); CSE (calibrator target sequence equivalents); CCE (calibrator cell equivalents); CGE (calibrator genome equivalents)
- Standardization of results in updated EPA Methods and correspondence with RWQC values (Enterococci)
  - Points to remember:
    - TSC are what are detected by qPCR. Other reporting units (CSE, CCE CGE) reflect efforts in the EPA methods to control for recovery of TSC from bacterial cells
    - CCE and CGE do not necessarily = cells (or CFU) in water samples (but current EPA RWQC values for Enterococci are based on CCE)
Real-Time PCR Quantification

- Target sequence copies increase exponentially (i.e. ~ double) during early cycles of PCR amplification. In real-time PCR, more copies = more fluorescence
- QPCR is based on this predictable increase in fluorescence during early cycles: i.e. the linear relationship between log10 target sequences in a reaction and number of cycles needed to reach a threshold where fluorescence first becomes detectable above background and is still increasing exponentially
- Quantitative Cycle threshold (C$_T$ or C$_q$) is the number of cycles required to reach the threshold
- If DNA standards with known target sequence quantities are available, a standard curve of their C$_T$ values can be used to estimate absolute quantities of target sequences in unknown test samples

Fluorescence threshold = 30 fluorescence units in this example
*Cycle threshold: Cycle # at which growth curve crosses 30
Absolute quantification of unknown target sequences using DNA standards

Standard Curve

Regression Formula: \( y = -3.417x + 38.071 \)

Test Sample \( C_T \): 27.5
Comparative C\textsubscript{T} Calibration Models

• Used to determine relative quantities of target sequences in different samples (no assumptions about absolute quantities).

• Results expressed as ratios or fold-differences in TSC quantities in test samples compared to a designated calibrator sample.

• Ability to normalize for differences in total DNA recovery in calibrator and test samples by comparing C\textsubscript{T} values of a reference target sequence expected to be present in equal amounts prior to sample processing.

• Comparative C\textsubscript{T} models are widely used for measuring changes in gene expression.

(Applied Biosystems, User Bulletin #2, 1997
The “EPA” calibration model

- The calibration model used in all EPA qPCR methods for general fecal indicator bacteria (FIB) is a hybrid of absolute and relative quantification approaches:
  - Absolute DNA standards are used to determine the mean and standard deviation of TSC recoveries from initial extracts of multiple calibrator samples (containing cells of representative strains of Enterococcus, E. coli, or Bacteroidales).
  - The ratios of TSC quantities recovered from test samples, compared to ongoing calibrator samples analyzed in parallel, are determined by the comparative $C_T$ model
    - Ongoing calibrator sample $C_T$ measurements must fall within a range established from std deviations of the initial calibrator samples
  - Ratios are multiplied by the mean absolute TSC value from the initial calibrator samples to give CSE in all updated methods.
    - Original Method 1611 model multiplied ratios by absolute cell counts in the calibrator samples
  - CSE can be converted to CCE (enterococcus) or CGE (E. coli or Bacteroidales) using applicable TSC to cell or genome ratios (slide 10).
• Expression of the model:

  – comparative $C_T$ ratio$_{\text{test to calibrator}} = AF^{(-\Delta\Delta C_T)}$
  – $CSE = \text{ratio} \times \text{calibrator TSC (from standard curve)}$

• where:

  – Amplification factor (AF) is determined from the slope of the standard curve from DNA standards by the formula: $10^{(1/\text{slope})}$
  – $\Delta\Delta C_T$ is determined by subtracting $C_T$Ref from $C_T$FIB for both calibrator and test samples ($= \Delta C_T$) and then subtracting Calibrator $\Delta C_T$ from Test sample $\Delta C_T$
    • $C_T$Ref is from a sample processing control (salmon DNA) PCR assay
    • $C_T$FIB is from the Enterococcus, E.coli or Bactoidales PCR assay
Challenges for standardized implementation of EPA QPCR Methods

- Variability in TSC/cell from different sources of calibrator cells can cause different CCE estimates
  - Demonstrated in several studies using different sources of cells to prepare calibrator samples (Sivaganesan, J. Micro. Meth. 2011; Cao, Water Res. 2012)
  - An impetus for moving from a cell count to a TSC based calibration model in the updated Methods

- Need for accurate DNA standards to determine TSC in calibrator samples

- Need to relate TSC in calibrator samples to NEEAR study calibrator samples in order to relate Enterococcus CCE values to RWQC values
10

Standardization of results in EPA QPCR Methods

- Updated EPA Methods 1609.1, 1611.1 and draft Method C require estimation of TSC in calibrator sample extracts according to the model described in this presentation
- Plasmid DNA standards with concentrations that have been corroborated at EPA by digital PCR analysis are currently available for these methods
- Enterococci CSE/test sample are divided by 15 (overall mean TSC/calibrator cell estimate from the NEEAR Epi studies – Haugland, J. Micro. Meth., 2014) in Methods 1609.1 & 1611.1 to obtain CCE that can be compared to RWQC values
- E. coli CSE/test sample are divided by 7 (reported copy number of 23S rDNA gene target sequences / E. coli genome) in draft Method C for E. coli to obtain CGE
- Draft method B for Bacteroidales has not been updated, but will probably use the same approach as Method C when updated
Standardization of results in EPA QPCR Methods

- Updated EPA Methods 1611.1 and 1609.1 and template Excel spreadsheet for performing standardized calculations of qPCR results according to these methods are available at new website:
  - [http://www2.epa.gov/cwa-methods/other-clean-water-act-test-methods-microbiological](http://www2.epa.gov/cwa-methods/other-clean-water-act-test-methods-microbiological)
  - Updated Draft Methods B & C are presently not available at this site, pending further testing of these methods. Draft Method C and template spreadsheet have been released on a limited basis.